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# Tachykinin activation of human alveolar macrophages in tobacco smoke and sarcoidosis: a phenotypical and functional study

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**Summary** Substance P (SP) and neurokinin A (NKA), which exert bronchoconstrictor effects on human airways, are known to interact with inflammatory and immune cells, including monocyte macrophages. We have evaluated the effects of SP, NKA and the NK<sub>2</sub> selective agonist [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10) on alveolar macrophages (AM) isolated from 4 healthy smokers and 4 non-smoker active pulmonary sarcoid patients. An accumulation of activated mononuclear phagocytes, as well as elevated angiotensin-converting enzyme (ACE) activity, has been evidenced in both clinical conditions. The phenotype of AMs in the studied subjects was characterized by an elevated expression of CD68+, HLA-DR+ and CD14+, CD14+ being significantly less in sarcoidosis as compared to smokers. SP, NKA and the NK<sub>2</sub> selective agonist evoked superoxide anion (O<sub>2</sub><sup>-</sup>) production in AMs obtained from sarcoid patients or healthy smokers. While SP acted in a non-dose-dependent manner in both conditions, NKA and [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10) evoked a dose-dependent respiratory burst (ED<sub>50</sub> = 0.25 and 0.26 nM, respectively) in smokers, but not in sarcoidosis. The more marked phenotypical expression correlated well with the ability of NK<sub>2</sub> receptors to activate AMs in smoker subjects.

## INTRODUCTION

Pulmonary sarcoidosis is a multisystemic granulomatous disease of unknown etiology mainly affecting lung interstitium, characterized by the presence of multiple non-caseating granulomas in affected tissues.<sup>1</sup> In this disease the preeminent immunological abnormality is an accumulation of activated CD4+ T lymphocytes at the site of disease activity, which in the respiratory tract is represented by a CD4+ lymphocytic alveolitis.<sup>2</sup> A central role in both the recruitment and activation of CD4+

lymphocytes within alveolar spaces is played by alveolar macrophages (AMs).<sup>3</sup> These cells, derived from circulating monocytes<sup>4</sup> and accumulated within alveolar structures during sarcoid alveolitis, show morphological, phenotypical and functional characteristics of mature, highly differentiated and activated mononuclear phagocytes,<sup>3</sup> producing and releasing increased amounts of proinflammatory cytokines and oxygen-derived free radicals as immune response to unknown antigen(s).<sup>3</sup> Another condition in which an accumulation of activated mononuclear phagocytes is usually observed is the macrophage alveolitis occurring in smoker subjects; in smokers a morpho-phenotypical and functional pattern of AMs as the one observed in sarcoidosis has been described to be associated with a not significantly increased number of AMs.<sup>5</sup>

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AMs are a heterogeneous cell population, differing in morphological, phenotypical and functional characteristics in both healthy subjects and patients.<sup>3-5</sup> By employing bronchoalveolar lavage (BAL), discontinuous gradient centrifugation or monoclonal antibodies, some interesting relationships between AM structure, phenotype and function have been evidenced in bronchial asthma as well as in pulmonary tuberculosis: large, hypodense cells are activated, mature mononuclear phagocytes showing an increased oxidative burst.<sup>6-8</sup> Unfortunately these findings are not homogeneously present in all evaluated conditions; for example, Sandron et al<sup>9</sup> failed to demonstrate any relationship between different AM subpopulations and their activity in both healthy subjects and patients with active pulmonary sarcoidosis. According to Hinman et al,<sup>10</sup> AMs from sarcoid patients or smokers share another common feature: both present an elevated angiotensin-converting enzyme (ACE) activity. However, this functional characteristic of AMs was also not observed by Gupta et al<sup>11</sup> who failed to demonstrate an increase of ACE levels in BAL fluid from smokers. ACE, together with neutral endopeptidase and aminopeptidase, is able to degrade tachykinins, a family of peptides with the same C-terminal sequence Phe-X-Gly-Leu-Met-NH<sub>2</sub>.<sup>12</sup> Three types of tachykinin receptors, namely NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub>, are described to preferentially interact with substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), respectively.<sup>12</sup> SP and NKA, which are co-localized to sensory afferent C-fibers and released locally by an axon reflex, are deeply involved in lung pathophysiology.<sup>12</sup> Relationships between these nervous fibers and tachykinin levels within lung tissues are underlined by the lack of tachykinin immunoreactive nerves in transplanted lungs and by the reduction of SP levels in BAL fluid from transplanted lungs.<sup>13,14</sup> Besides inducing bronchoconstriction in vivo and in vitro, enhancing plasma exudation and mucous secretion, tachykinins exert modulatory effects on cells involved in inflammatory and immune processes.<sup>12</sup> SP degranulates mast cells, stimulates both DNA and protein synthesis from human T lymphocytes and exerts priming effects on neutrophils.<sup>15,16</sup> Lotz et al<sup>17</sup> demonstrated that NKA and SP (the former being more potent) evoke the release of pro-inflammatory cytokines from human circulating monocytes. Brunelleschi et al<sup>18</sup> reported that tachykinins activate guinea-pig AMs: the order of potency (NKA > SP > NKB) and the ability of an NK<sub>2</sub> receptor agonist to evoke superoxide anion production and eicosanoid release from these cells indicate that guinea-pig AMs present tachykinin NK<sub>2</sub> receptors mainly.<sup>18,19</sup> Therefore, we decided to evaluate whether or not SP, NKA and a selective NK<sub>2</sub> receptor agonist were able to induce superoxide anion production from AMs of sarcoid patients or human smokers.

## MATERIALS AND METHODS

### Study population

Four patients, 3 males and 1 female, aged between 28 and 46 years, non-smokers, with newly diagnosed pulmonary sarcoidosis, were studied. Diagnosis was done on the basis of clinical, laboratory and chest X-ray data, and confirmed by histological findings on specimens from transbronchial lung biopsies during bronchoscopy after bronchoalveolar lavage was done in the lobe. Histological analysis of biopsy specimens showed the presence of typical sarcoid (non-caseating granuloma with giant cells and no necrosis) in all samples. All sarcoid patients had an active disease, as defined by employing standard criteria: clinical (fever, chest pain, dyspnoea on exertion, cough), scintigraphic (positivity of <sup>67</sup>gallium lung scan), biological (elevated serum angiotensin-converting enzyme [sACE] levels) and lung functional (at least reduced resting carbon monoxide diffusion, DICO) tests. None of the patients had a medical history of asthma, and pulmonary function tests excluded actual obstruction. No patients had received steroids or other therapy at the time of the study. The other group was composed by 4 healthy smoker subjects (3 males and 1 female) aged between 18 and 63 years. This study and the research protocol were approved by the Ethical Committee of the University of Turin.

### Bronchoalveolar lavage and cell preparation

Bronchoalveolar lavage (BAL) was performed as previously described.<sup>20</sup> Briefly, informed consent was obtained from each subject; after pretreatment with parenteral atropine sulphate (0.5 mg), airways were anesthetized with 2% lidocaine. A fiberoptic bronchoscope (Olympus BF-1T10) was advanced and wedged into the middle lobe under direct visualization. Lavage was carried out with 200 ml of prewarmed (37°C) sterile saline solution in ten 20-ml aliquots with immediate gentle vacuum (syringe) aspiration after each injection. The total amount of recovered fluid was measured (always >60% of injected saline); the first recovered aliquot was separately examined for cytological and bacteriological studies and the other ones pooled. Immediately after lavage the fluid was filtered through two layers of sterile surgical gauze. Cells were separated from the lavage fluid by centrifugation in a refrigerated centrifuge (4°C, 20 min, 900 rpm) and washed in phosphate-buffered salt solution (PBS; Serva). Total cell count and viability evaluation (Trypan blue dye exclusion test; always >95%) were performed on a Burkert haemocytometer, differential cell count was done on Diff-Quick (Don Baxter) stained cytopsin smears, counting at least 400 cells.

Immediately before BAL premedication, peripheral blood samples were obtained from each studied subject by venipuncture; 10-ml vacuum sterile tubes (Vacutainer, Becton-Dickinson) were employed. The samples were allowed to clot; serum was gently removed after centrifugation (5 min, 2000 rpm) and stored at  $-80^{\circ}\text{C}$  until utilized for sACE level determinations.

### Phenotypical studies of alveolar cells

#### *Alveolar lymphocytes*

The whole cellular BAL pellet was washed twice in PBS, resuspended in sterile culture medium (RPMI 1640) and placed in flat bottom, multiwell polystyrene plates. After 1 h incubation at  $37^{\circ}\text{C}$  in humidified 5%  $\text{CO}_2$  atmosphere, non-adherent cells (mainly lymphocytes) were gently collected. Phenotypical analysis of these cells was carried out, after two additional PBS washings, by employing a panel of commercial available monoclonal antibodies (Table 1) and flow cytometry (FACSCAN, Becton-Dickinson). Standard staining procedures<sup>21</sup> and single or two-colour analysis with FACSCAN software were used (at least 5000 events for each sample were assumed and cells evaluated). Lymphocyte gates were obtained by using linear forward angle light scatter, the lymphocyte gates were validated by employing CD45-fluorescein-conjugated (panleucocyte) and CD14phycoerythrin-conjugated (monocyte) monoclonal antibodies.<sup>21</sup> The lymphocyte population was defined as  $\text{CD45}^{\text{bright}}\text{CD14}^{\text{negative}}$ ; more than 95% of all lymphocytes in each sample were contained within the boundaries of the lymphocyte light-scatter gate.

#### *Alveolar macrophages*

Adherent AMs were obtained after 1 h incubation at  $37^{\circ}\text{C}$  in humidified 5%  $\text{CO}_2$  atmosphere and used for

functional studies (see below). Adherent BAL cells, after removal of non-adherent ones by gentle washing with prewarmed sterile PBS, were detached by scraping with a plastic scraper. After three washings with sterile PBS, the removed adherent cells were resuspended at the final concentration of  $1 \times 10^5$  cells/ml in sterile PBS; thereafter, cytocentrifuge preparations were prepared from this cell suspension in a cytocentrifuge (Cytospin, Shandon, UK) at 500 rpm for 10 min. Phenotypical analysis of alveolar macrophages was carried out on these cytocentrifuge slides by employing leukocyte-specific monoclonal antibodies (MoAb) as listed in Table 1. As controls non-specific mouse IgG1, IgG2 and IgM (Dako) were used; staining with these negative controls was always negative. Briefly, cytocentrifuge preparations were fixed in ethanol (Merck) for 5 min and washed with PBS (one smear was employed for each MoAb) and incubated with horse non-immune anti-mouse serum (Dako) for 20 min. After washing three times with PBS, the slides were incubated for 1 h with the appropriate MoAb at the dilution suggested by the manufacturer. After washing three times with PBS, the slides were incubated for 1 h with a directly biotinylated second antibody at 1/200 dilution in PBS (Dako). After three washings with PBS, 1 h incubation with avidine-biotin complex conjugated with alkaline phosphatase (Dako) was performed; at last, after a washing with Tris-HCl (0.1 M, pH 8.2), fast red naphthol phosphate (with levamisole 1.25 mM) (Dako) was added ( $37^{\circ}\text{C}$ , 5 min) to develop a brilliant red staining of positive cells (this colour was chosen to avoid possible interferences with brownish or black cytoplasmic inclusions of alveolar macrophages). Positive cells were counted by evaluating at least 200 cells. Selected slides were stained for either acid phosphatase or non-specific esterase (alpha-naphthyl esterase). Counterstaining

**Table 1** Monoclonal antibodies employed in the phenotypical study of mononuclear alveolar cells

CD	Commercial	Cell/function
CD3	Anti-LEU4a (BD)	T-lymphocytes
CD4	Anti-LEU3a (BD)	Helper/inducer T-lymphocytes
CD8	Anti-LEU2a (BD)	Suppressor/cytotoxic T-lymphocytes
–	Anti-HLA-DR (BD)	II Class MHC, activated T-lymphocytes and macrophages
CD25	Anti-IL2 Receptor (BD)	IL-2 receptor, $\alpha$ chain; activated, proliferating T-lymphocytes
CD68	Anti CD68 (Dako)	Oxidative burst-related antigen
CD14	Anti-LEUm3 (BD)	LPS receptor
CD45	Anti-human Leukocyte (BD)	Pan leukocyte

Cluster designation (CD; when available), commercial definition and source, and characteristics of cells identified by monoclonal antibodies are contained in this table. BD: Becton Dickinson; Dako: Dako Patts.

was performed with hematoxylin (Merck). Finally, the slides were dehydrated in ethanol and embedded in mounting medium (Entellan Merck). The slides were evaluated independently by two investigators and at least 200 cells were counted for each marker on each slide.

#### Functional studies of alveolar macrophages: superoxide anion production

Adherent AMs ( $10^6$ /dish) were washed twice with PBS and then incubated in sterile culture medium (MEM without phenol red). Alveolar macrophages were pretreated for 15 min at 37°C with a cocktail of inhibitors: captopril, thiorphan and bestatin, all at 1  $\mu$ M. This concentration was demonstrated to block tachykinin-degrading enzymes.<sup>12,18</sup> Cells were then challenged with increasing concentrations of substance P (SP), neurokinin A (NKA) and [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10) (a selective NK<sub>2</sub> receptor agonist) for 30 min. The effects of tachykinins were compared with those evoked by the bacterial peptide *N*-formylmethionyl-leucyl-phenylalanine (FMLP).

Superoxide anion ( $O_2^-$ ) production was evaluated by the superoxide dismutase-inhibitable cytochrome C reduction, the absorbance changes being recorded at 550 nm in a Perkin Elmer spectrophotometer.  $O_2^-$  production was expressed as nmol cytochrome C reduced/ $10^6$  AMs/30 min, using an extinction coefficient of 21.1 mM.<sup>18</sup> To avoid interference with spectrophotometrical recordings of  $O_2^-$  production, monolayers were incubated with MEM without phenol red.

Experiments were performed in triplicate and control values (e.g. basal  $O_2^-$  production) were subtracted from all determinations. All results are expressed as means + SEM. Statistical analysis was performed by using Student's *t* test.

#### BALf concentration procedure

Each sample of BAL, after centrifugation and separation of the cellular pellet, was concentrated by employing a selective membrane for ultrafiltration with an adequate molecular weight cut-off (10 000 Da) and with low interaction with proteins (Amicon YM10) in a magnetic-stirred positive pressure (N) cell (Amicon Grace, Italy).

#### ACE level measurements in bronchoalveolar lavage fluid (IACE) and in serum (sACE)

Levels of ACE in both serum and 50-fold concentrated BALf were determined by commercially available (ACEcolor, Fujirebio Inc, Tokyo, Japan) colorimetric method, employing *p*-hydroxybenzoyl-glycyl-L-histidyl-L-leucine as substrate for ACE activity determination. The absorbance was

read at 505 nm, using distilled water as control. The cut-off value was 21.4 mU/ml for sACE and 30.7 mU/ml for IACE, respectively (mean values of healthy controls + 2 SD of the mean).

#### Chemicals

The compounds used and their sources were as follows: SP, NKA, [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10), bestatin (Peninsula); thiorphan, captopril, superoxide dismutase, cytochrome C type VI, Tris-HCl, FMLP (Sigma); PBS and RPMI 1640 (Serva); MEM without phenol red (Gibco).

## RESULTS

#### Phenotypical studies of alveolar cells

All samples from studied subjects showed no growth of Gram+ve or Gram-ve bacteria, mycobacteria or fungi (data not shown). Cytological examination of all BAL samples showed no presence of atypical cells (Papanicolau staining). More than 95% of recovered cells were viable in all studied samples. Total and differential cell count in patients with pulmonary sarcoidosis and in healthy smokers are shown in Table 2: in both healthy smokers and sarcoid patients an increase of total cell count was observed. Alveolar lymphocytosis was higher in the sarcoidosis group, whereas a marked increase of alveolar macrophages was observed in smokers (Table 2). Moreover, a mild increase of alveolar neutrophil percentage was observed only in smokers (Table 2).

Alveolar lymphocyte subsets and activation antigen expression on alveolar lymphocytes in both healthy smokers and sarcoid patients are shown in Table 3: in smoker subjects a normal CD4/CD8 ratio was observed, while in sarcoid patients the hallmark of an increased percentage of CD4+ cells and a significantly elevated CD4/CD8 ratio were found. As expected, the activation marker expression was particularly evident in the sarcoidosis group as evidenced by the results obtained by evaluating CD3+/HLA-DR co-expression and CD4+/CD25+ co-expression (Table 3). More than 90% of cells on cytospin slides were acid-phosphatase positive phagocytes reacting with non-specific esterase.

The phenotype of AMs in studied subjects is reported in Table 4: the great majority of AMs ( $96.25 \pm 2.3\%$ ) in smokers were CD68+ and a high percentage ( $83.75 \pm 2.3\%$  and  $67.5 \pm 7.7\%$ , respectively) of AMs also expressed CD14 and HLA-DR. In pulmonary sarcoid patients, the percentage of AMs expressing CD68+, although elevated ( $82 \pm 5\%$ ), was significantly less ( $P < 0.05$ ) than the one observed in smokers, as was the percentage of AMs expressing CD14+ ( $25.2 \pm 1.8\%$ ;  $P < 0.001$ ) (Table 4). Levels of ACE in both serum and BALf are recorded in Table 5: all sarcoidosis patients showed sACE and IACE levels significantly higher ( $P < 0.001$ ) than smoker subjects.

**Table 2** Total and differential cell count in BALf of studied subjects

Patients (age; sex)	Total cell ( $\times 10^5$ )	AM(%)	LY(%)	PMN(%)	EOS(%)
<b>Smokers</b>					
1 (54; M)	2.01	93	6	1	0
2 (63; M)	1.45	90	7	3	0
3 (50; M)	5.20	90	5	5	0
4 (18; F)	5.22	93	3	4	0
Means $\pm$ SEM	3.47 $\pm$ 1	91.5 $\pm$ 0.8	5 $\pm$ 1	3.25 $\pm$ 0.8	0
<b>Sarcoidosis</b>					
5 (43; M)	1.61	64	35	0	1
6 (46; M)	2.89	92	6	2	0
7 (29; M)	4.54	59	40	1	0
8 (28; F)	2.37	70	29	1	0
Means $\pm$ SEM	2.85 $\pm$ 0.6	71.2 $\pm$ 7.2*	27.5 $\pm$ 7.5*	1 $\pm$ 0.4*	0.25 $\pm$ 0.25

Data are given as total cell number/ml BAL  $\times 10^5$  and as percentage of total cell population (differential). AM = alveolar macrophages, LY = alveolar lymphocytes; PMN = alveolar neutrophils; EOS = alveolar eosinophils. \* $P < 0.05$  vs smokers.

### Superoxide anion production from alveolar macrophages

Basal values ( $O_2^-$  production from unstimulated cells) in smoker subjects and sarcoid patients were  $12.04 \pm 0.4$  ( $n = 4$ ) and  $4.02 \pm 0.86$  ( $n = 4$ ;  $P < 0.01$ ) nmol cytochrome C reduced/ $10^6$  AMs/30 min, respectively. These values were subtracted from those observed after tachykinin or FMLP challenge to obtain the net  $O_2^-$  production.

The bacterial peptide FMLP, used at  $10^{-7}$  M (a near maximal concentration), produced  $4.12 \pm 0.4$  ( $n = 4$ ) and  $7.32 \pm 1.9$  ( $n = 4$ ) nmol cytochrome C reduced/ $10^6$

AMs/30 min in smoker subjects and sarcoid patients, respectively.

When challenged with SP, NKA or the selective  $NK_2$  agonist [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10), AMs from smokers presented a significant  $O_2^-$  production (Fig. 1). However, while SP ( $10^{-10}$  to  $10^{-6}$  M) acted with a non-dose-dependent pattern, NKA ( $10^{-12}$  to  $10^{-6}$  M) displayed a dose-dependent effect, maximal activation ( $9.6 \pm 2.6$  nmol cytochrome C reduced/ $10^6$  AMs/30 min;  $n = 4$ ) being achieved at  $10^{-7}$  M and  $ED_{50}$  value being 0.25 nM (Fig. 1). The  $NK_2$  selective agonist evoked a dose-dependent  $O_2^-$

**Table 3** Alveolar lymphocyte subsets and activation antigen expression in smoker subjects and in non-smoker active pulmonary sarcoidosis

Patients	CD3+	CD4+	CD8+	CD4+/CD8+ ratio	CD3+/HLADR+ co-expression	CD4+/CD25+ co-expression
<b>Smokers</b>						
1	95.0	72.1	20.7	3.43	14.5	1.5
2	92.8	48.6	46.4	1.04	7.5	0.3
3	93.8	56.3	36.8	1.52	26.3	1.0
4	95.0	45.9	44.3	1.03	6.8	1.2
Means $\pm$ SEM	94.15 $\pm$ 0.5	55.5 $\pm$ 5	37.05 $\pm$ 5	1.75 $\pm$ 0.5	13.8 $\pm$ 4.5	1 $\pm$ 0.25
<b>Sarcoidosis</b>						
5	89.0	62.0	14.6	4.25	15.5	2.9
6	96.4	73.4	18.3	4.01	36.5	4.4
7	96.1	81.9	11.6	7.06	27.4	3.6
8	97.1	88.0	7.6	11.05	41.7	3.3
Means $\pm$ SEM	94.8 $\pm$ 1.9	76.3 $\pm$ 5.6 *	13 $\pm$ 2.2 *	6.7 $\pm$ 1.7 *	30.3 $\pm$ 5.7 *	3.5 $\pm$ 0.3 **

Positive cells are expressed as percentage of total alveolar lymphocytes. \*  $P < 0.05$  vs smokers; \*\*  $P < 0.01$  vs smokers.

**Table 4** CD68, HLA-DR and CD14 expression on alveolar macrophages in smoker subjects and in non-smoker active pulmonary sarcoidosis

Patients	CD68 +	HLA-DR +	CD14 +
Smokers			
1	100	80	65
2	90	90	90
3	100	85	55
4	95	80	60
Means $\pm$ SEM	96.2 $\pm$ 2.3	83.7 $\pm$ 2.3	67.5 $\pm$ 7
Sarcoidosis			
5	1.61	64	22
6	2.89	92	30
7	4.54	59	26
8	2.37	70	23
Means $\pm$ SEM	82 $\pm$ 5 *	94 $\pm$ 2.3 *	25.2 $\pm$ 2 **

Positive cells are expressed as percentage of total alveolar macrophages. \*  $P < 0.05$  vs smokers; \*\*  $P < 0.01$  vs smokers.

production, with maximal effects at  $10^{-6}$  M and an  $ED_{50} = 0.26$  nM (Fig. 1).

As depicted in Figure 2, SP, NKA and [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10) induced  $O_2^-$  production in a non-dose-dependent fashion in AMs from sarcoid patients. The amount of  $O_2^-$  production evoked by NK<sub>2</sub> receptor stimulation was less ( $P < 0.05$ ) than the ones observed in smokers, while SP exerted similar effects (Fig. 2).

When the respiratory burst was evaluated as a percent of control values, an interesting feature emerged. In smoker subjects, NKA-evoked  $O_2^-$  production ranged between 110 and 180% of the control value, at  $10^{-12}$  M and  $10^{-7}$  M, respectively, while in sarcoid patients NKA-evoked

$O_2^-$  production increased about 2-fold for all the concentrations evaluated (data not shown). SP-evoked  $O_2^-$  production increased to about 140–150% of the control value in smokers and 230–240% of the control value in sarcoid patients for all the concentrations evaluated (data not shown). [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10) induced a higher respiratory burst ranging from 120% of the control value at  $10^{-11}$  M to 180% at  $10^{-6}$  M in smokers and about 220–240% of the control value in sarcoid patients (data not shown). FMLP-evoked  $O_2^-$  production was about 130% of the control value in smokers and 280% in sarcoid patients.

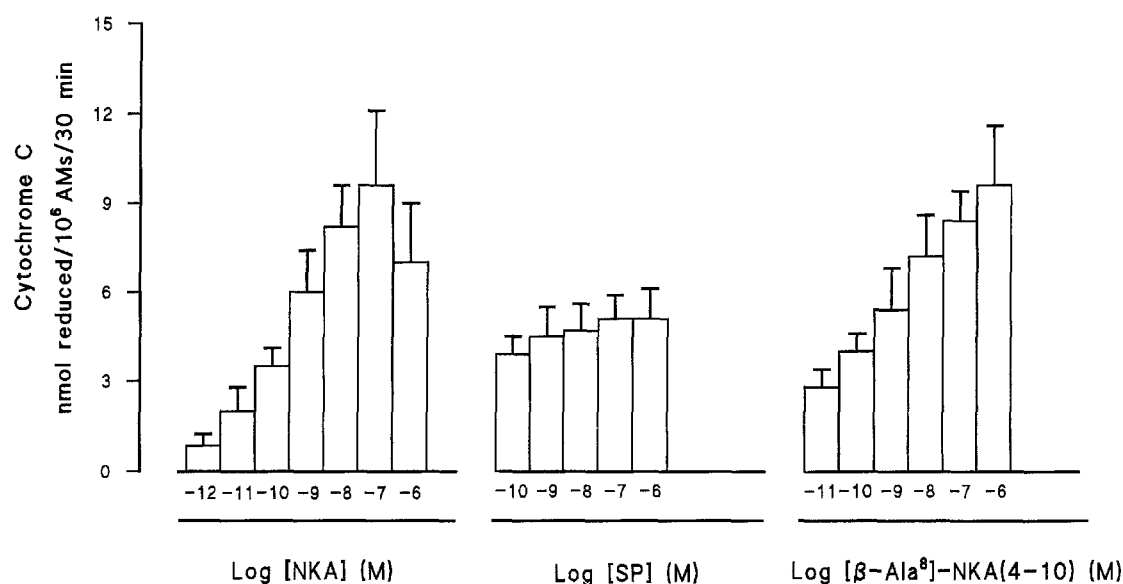
## DISCUSSION

This study demonstrates that mammalian tachykinins SP and NKA, as well as the selective NK<sub>2</sub> agonist [ $\beta$ -Ala<sup>8</sup>]-NKA(4–10), induce  $O_2^-$  production in AMs obtained from sarcoid patients or healthy smokers. As far as SP (which represents the major endogenous agonist for the tachykinin NK<sub>1</sub> receptor) is concerned,  $O_2^-$  production increased to more than 230% of the control value in sarcoid patients, while in smokers the respiratory burst was about 150% of the control value. However, while SP acted dose-independently in both conditions, NKA and the NK<sub>2</sub> selective agonist evoked a dose-dependent respiratory burst in smokers, but not in sarcoid patients. The functional study was performed in the presence of a cocktail of inhibitors (thiorphan, captopril and bestatin) of tachykinin-degrading enzymes (neutral endopeptidase [NEP], ACE and aminopeptidase, respectively), since it is known from the literature that the activity of NEP and ACE represents an important factor in determining the effects of tachykinins.<sup>12,22</sup> Moreover, the activity of both degrading enzymes could

**Table 5** ACE levels in BAL fluid (IACE) and serum (sACE)

Patient	sACE	IACE
Smokers		
1	4.3	8.1
2	10.2	14.5
3	6.5	11.6
4	8.1	12
Means $\pm$ SEM	7.25 $\pm$ 1.2	11.52 $\pm$ 1.3
Sarcoidosis		
5	28.4	58.1
6	32.5	64.2
7	41.1	71.4
8	35.4	59.6
Means $\pm$ SEM	34.3 $\pm$ 2.7 *	63.3 $\pm$ 2.9*

Data are expressed as mU/ml serum and mU/ml unconcentrated BAL fluid. \* $P < 0.001$  vs smokers.

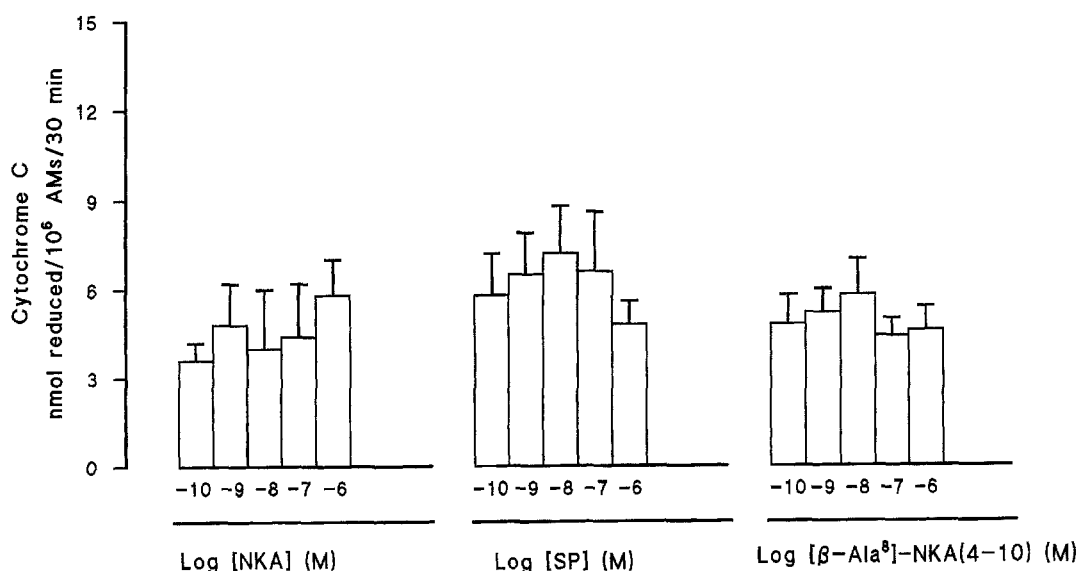


**Fig. 1** Effect of neurokinin A (NKA), substance P (SP) and the NK<sub>2</sub> selective agonist [ $\beta$ -Ala<sup>8</sup>]-NKA(4-10) on superoxide anion production in alveolar macrophages obtained from healthy smokers. Cells were pretreated with a cocktail of inhibitors (see Methods) and then challenged with tachykinins. Means  $\pm$  SEM;  $n = 4$ .

be modified in the clinical conditions we evaluated. Hinman et al<sup>10</sup> reported that human AMs 'normally' contain ACE activity and that this activity increased five times in AMs obtained by the three sarcoid patients they evaluated, while a two-fold increase was detected in AMs from 11 smokers. Dusser et al<sup>23</sup> demonstrated that, in the

guinea-pig, cigarette smoke enhanced SP-induced bronchoconstriction by inactivating airway NEP.

SP and NKA are present in human lung and have been detected and measured in BAL, as well as NEP and ACE.<sup>22</sup> so, we thought to evaluate the effects of tachykinins on AMs in the presence of inhibitors. To our knowledge,



**Fig. 2** Effect of neurokinin A (NKA), substance P (SP) and the NK<sub>2</sub> selective agonist [ $\beta$ -Ala<sup>8</sup>]-NKA(4-10) on superoxide anion production in alveolar macrophages obtained from sarcoid patients. Cells were pretreated with a cocktail of inhibitors (see Methods) and then challenged with tachykinins. Means  $\pm$  SEM;  $n = 4$ .



only Pujol et al<sup>24</sup> have been interested in evaluating tachykinin effects on human AMs, while many investigators have determined tachykinin effects in guinea-pig AMs<sup>18,19,25,26</sup> or human monocytes.<sup>17,27,28</sup> Pujol et al<sup>24</sup> evaluated the ability of SP, at concentrations ( $10^{-7}$  M to  $10^{-4}$  M) higher than the ones we have used in this study, to stimulate TXB<sub>2</sub> release in AMs from six normal subjects and seven asthmatic patients, but they found a significant TXB<sub>2</sub> release only in one asthmatic patient and no stimulation in normal subjects. We have not evaluated control healthy subjects, as we were more interested, in this first phase of our work, to look at possible tachykinin effects in pathological conditions. The results obtained by measuring spontaneous O<sub>2</sub><sup>-</sup> production, which is significantly enhanced in smokers' AMs as compared to sarcoid patients' AMs, are in good agreement with published data.<sup>8, 29, 30</sup>

The ability of tachykinins to stimulate AMs in active pulmonary sarcoidosis and tobacco smoke related alveolitis reflects two different clinical conditions, both characterized by an increased number of activated AMs. Otherwise, the observed differences of the phenotypical pattern of AMs, showing a more marked expression of CD68, HLA-DR and CD14, respectively, related to the presence of mononuclear phagocytes involved in the oxidative burst (CD68),<sup>31</sup> in antigen presentation (HLA-DR)<sup>32</sup> and in cytokine production by LPS (lipopolysaccharide) receptor (CD14)<sup>33</sup> that we found in smoker group vs sarcoidosis patients, correlated well with the ability of tachykinins (and especially NK<sub>2</sub> receptor stimulation) to activate AMs in our patients.

Recently, Tomaki et al,<sup>34</sup> in a study designed to investigate the reasons for the development of cough in a small percentage (10–15%) only of patients treated with ACE inhibitors, suggested that the effects of SP could be related to a receptor-mediated priming of the airways, since cough-positive patients receiving a repeated dose of enalapril after discontinuation of the therapy, presented the symptoms again, while SP levels in induced sputum were similar to the ones measured whilst off the medication.<sup>34</sup> In patients with chronic bronchitis, the increased levels of SP in induced sputum have been claimed to activate inflammatory mechanisms leading to local airway damage.<sup>35</sup> In sarcoidosis, a disease in which an increase in both sACE and IACE levels has been described as a consequence of the enhanced secretory activity of AMs,<sup>36,37</sup> a particular local (alveolar) condition may develop: high concentrations of tachykinin degrading enzyme (e.g. ACE) could reduce local levels of SP, whereas the same mechanism could not be effective in smokers. Consequently, although our study refers to a small number of patients only, we could hypothesize that different responses to tachykinin stimulation of AMs obtained from healthy

smokers (showing lower levels of IACE as compared to sarcoid patients) may be related to a different priming of AMs, largely mediated by the different levels of ACE activity within the alveolar microenvironment. The activation pattern of AMs following tachykinin stimulation we observed in smokers is in keeping with the observation of enhanced SP content in sputum from chronic bronchitis patients. The increase of tachykinin-evoked AM oxidative burst we observed in smokers could be considered to represent the result of an AM priming (which is highly possible in patients without a significant increase of alveolar ACE levels). In keeping with this hypothesis is the observation that the prevalence of pulmonary sarcoidosis is reduced in smoking population.<sup>38</sup> In addition, among patients with pulmonary sarcoidosis, the smoker ones show higher sACE levels and lower CD4/CD8 ratio in BAL, related to an increase in CD8+ lymphocytes.<sup>38</sup>

In conclusion, tachykinins may represent another group of mediators capable of modulating lung damage in different conditions by their ability to activate AMs; the presence of varied levels of ACE activity in alveolar spaces observed in smokers and in active pulmonary sarcoidosis seems to influence this activation pathway.

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